Chapter 5

Blockade of paraventricular hypothalamic alpha-adrenoceptors impairs the counterregulation to hypoglycemia

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A complicated system of counterregulatory responses exists to detect and counteract insulin-induced hypoglycemia. These counterregulatory responses are likely to be regulated by the brain in response to changes in blood glucose levels and other factors. It has been shown that noradrenaline levels in the hypothalamus rise during hypoglycemia. To investigate whether this noradrenergic signal is indeed crucial for the activation of the counterregulatory responses, rats received selective antagonists to alpha-adrenoceptors in the paraventricular hypothalamus, and were subjected to hypoglycemia by intravenous infusion of insulin, while counterregulatory responses were monitored. It was shown that blockade of the noradrenergic signal resulted in impaired counterregulatory responses, in particular the adrenaline and noradrenaline responses. Glucose levels decreased more than in the control rats, and plasma corticosterone levels were increased. The data suggest that noradrenergic neurotransmission through the paraventricular hypothalamus plays an important role in the sympathoadrenal counterregulation to insulin-induced hypoglycemia.

Introduction

Hypoglycemia, a common side-effect in insulin-treated diabetes, is counterregulated by a variety of responses, aimed at restoring normoglycemia. There are several lines of evidence that show that the brain is involved in this counterregulation against hypoglycemia. First, the brain – and especially the hypothalamus – is known to regulate energy balance (11, 20, 25, 28). Second, the brain receives information from multiple glucose-sensitive sites, both inside and outside the brain (4, 14, 18, 29). Third, the hypothalamus is activated during hypoglycemia (13), and both lesions (3), surgical damage (21) and inactivation (7) of hypothalamic nuclei have been shown to attenuate counterregulatory responses to hypoglycemia.

From studies about the neurochemical nature of the involved neuronal networks, it appears that in particular the noradrenergic circuits may play an important role in the regulation of metabolism (26) and the counterregulatory responses to hypoglycemia. Noradrenergic projections from the hindbrain to the hypothalamus are involved in the counterregulatory responses (17), and in the previous chapter we showed that noradrenaline levels in the hypothalamus are indeed elevated during hypoglycemia, just as they do during 2-DG-induced glucoprivation (1). Within the hypothalamus, especially noradrenergic neurotransmission in the paraventricular hypothalamic nucleus (PVN) may play a major role in regulating the counterregulatory responses to hypoglycemia. The PVN is an important neuronal integration center, with direct connections to the sympathetic nervous system, the parasympathetic nervous system, and the hypothalamo-pituitary-adrenal system; and noradrenergic systems in the PVN have been shown to be important in the regulation of blood glucose (7, 24) and food intake (8, 12).

The current study was designed to investigate the contribution of noradrenergic neurons projecting to the paraventricular hypothalamus to the counterregulatory responses to hypoglycemia. We focused on the role of α-adrenergic receptors, based on a pilot study as

well as data from the literature (2, 8). Therefore we subjected rats to insulin-induced hypoglycemia while α_1- or α_2-adrenergic neurotransmission in the PVN was inhibited, and measured the counterregulatory responses glucagon, adrenaline, noradrenaline, and corticosterone.

Methods

Animals and surgery

Male Wistar rats were used, weighing 346 ± 3 gram at the beginning of the experiments, and housed singly under standard conditions (lights on from 08:00-20:00, temperature 21 ± 1 °C) with ad lib access to water and food (standard RMH chow, Hope Farms, Woerden, The Netherlands) unless otherwise stated. The animals were frequently handled and weighed.

For stress-free blood sampling and i.v. infusions in freely-moving animals, all rats were fitted under halothane/N_2O inhalation anesthesia with two permanent silicone catheters (Medica BV, Den Bosch, The Netherlands), one for blood sampling and one for infusions. The catheters were inserted via the jugular vein, with the catheter tips ending in the superior vena cava just before the right atrium (23). In addition, two stainless steel cannulas (dimensions 0.15 mm ID, 0.30 mm OD) were bilaterally inserted into the brain, aimed at the left and right paraventricular nuclei (stereotactic coordinates: anterioposterior 1.6 mm from bregma, lateral 0.5 mm from midline, and dorsoventral 7.4 mm from dura, according to the brain atlas of Paxinos & Watson (16); see *Figure 1*), and secured to the skull by screws and dental cement. The cannulas were kept closed by steel wire inserts (0.12 mm OD) and protected by a metal cap. After surgery, the rats were allowed at least two weeks for recovery, and were habituated to the experimental conditions.

The experimental procedures were approved by the Animal Experiments Committee of the University of Groningen.

Experimental design

On the experiment day, food was removed after the lights went on. The venous catheters and brain cannulas were connected to polyethylene tubings to enable remote venous access and brain infusion in freely-moving animals.

After a resting period of at least one hour, two baseline blood samples were taken, with a 10-minute interval. Then, a bilateral infusion into the PVN was started, which ran at a rate of 0.15 µl/min over a period of 8 minutes and 20 seconds, resulting in a total volume of 1.25 µl infused into each PVN. Control treatment (n=9) consisted of 1.25 µl sterile artificial cerebrospinal fluid (aCSF; 0.4 mM NaH_2PO_4, 25 mM $NaHCO_3$, 122 mM NaCl, 3.1 mM KCl, 1.3 mM $CaCl_2$, and 1.2 mM $MgCl_2$; pH 7.4). To inhibit α_1-adrenergic transmission, the selective α_1-adrenoceptor antagonist prazosin (Sigma-Aldrich Chemie, Zwijndrecht, The Netherlands) was infused in a dose of 0.05 µg in 1.25 µl aCSF (n=8). Inhibition of α_2-adrenergic transmission was achieved by infusion of the selective α_2-adrenoceptor

antagonist yohimbine (Sigma-Aldrich Chemie), infused in a dose of 0.25 µg in 1.25 µl aCSF (n=9).

Another blood sample was taken 15 minutes after the start of the PVN infusion, and another five minutes later, at the time point defined as t=0, a 90-minute intravenous infusion of 120 mU.kg^{-1}.min^{-1} insulin was started (Velosulin, Novo Nordisk Farma, Alphen a/d Rijn, The Netherlands). The dose of insulin was based on the previous studies in our laboratory, where this dose produces significant counterregulatory responses (Chapter 2). Blood samples were regularly taken throughout the infusion period to measure changes in glucose levels and in the counterregulatory responses glucagon, adrenaline, noradrenaline, and corticosterone.

After the last sample (at t=90 minutes), the insulin infusion was stopped. Half an hour later, food was returned to the animals, and total food intake during the following hour was determined (i.e., from t=120 to t=180 minutes).

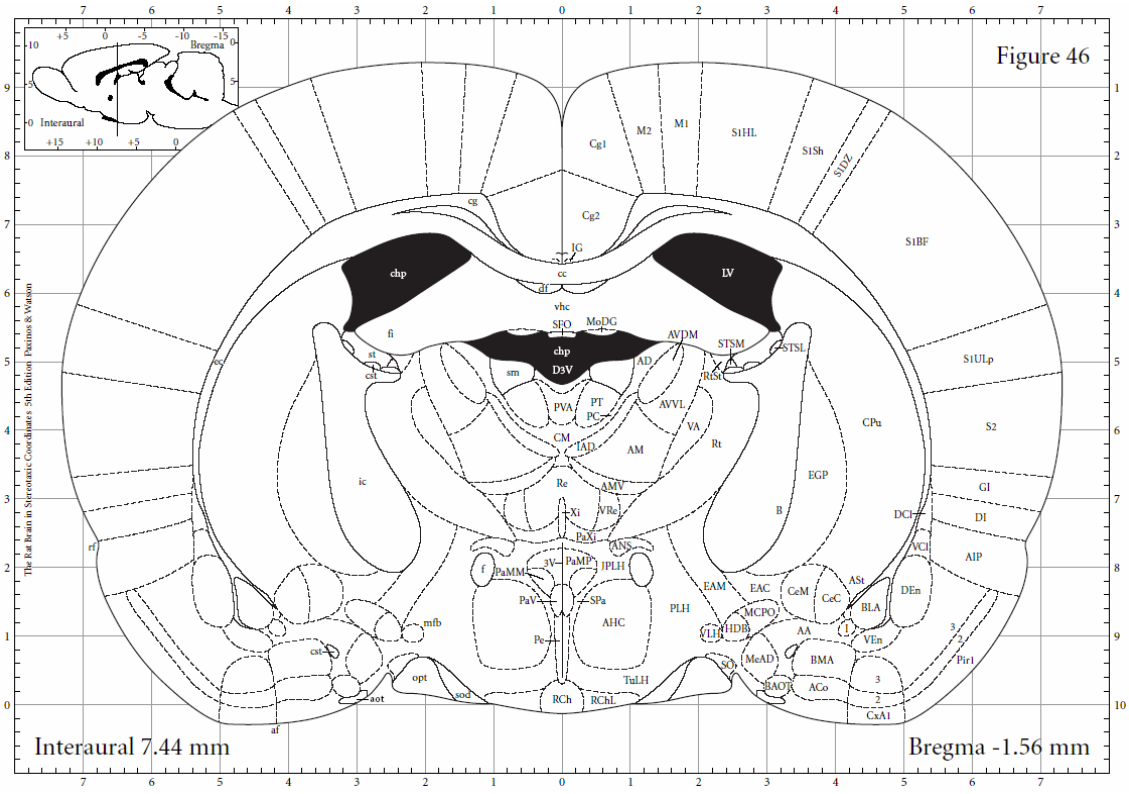

Figure 1. Coronal section of the brain at -1.56 mm from bregma, according to the rat brain atlas of Paxinos & Watson (15). The paraventricular hypothalamus is labeled PaMM, PaMP, and PaV.

Analysis

The blood samples were collected in tubes containing EDTA and aprotinin, and kept chilled during the experiment. Afterwards, 50 µl blood was removed to determine glucose levels (10), while the remaining blood was centrifuged for 15 minutes at 2600 G and 4 °C. Plasma aliquots were frozen until determination of glucagon (Glucagon RIA Kit, Linco

Research, St. Charles, MO, USA), adrenaline and noradrenaline (HPLC with electrochemical detection (22)), and corticosterone (HPLC with UV detection (6)).

Results are reported as average ± SEM (standard error of the mean). Statistical differences were determined with ANOVA or t-test (paired where relevant). The significance level was set to $p<0.05$.

Results

The effects of paraventricular hypothalamic α-adrenergic blockade on the counterregulatory responses to insulin-induced hypoglycemia are depicted in *Figure 2*.

In the control experiment (vehicle infusion into the PVN), intravenous infusion of 120 $mU.kg^{-1}.min^{-1}$ insulin during 90 minutes rapidly decreased blood glucose levels, to a final level of 3.1 ± 0.2 mM. This hypoglycemia was accompanied by significant rises in glucagon, adrenaline, and noradrenaline levels (final levels 140 ± 6, 1598 ± 611 and 350 ± 27 pg/ml; $p<0.05$ vs baseline levels). Glucagon responded first, reaching statistical significance from t=15 minutes after the start of the insulin infusion, while adrenaline and noradrenaline were significantly elevated from t=60 onwards. Corticosterone levels were already increased during vehicle infusion into the PVN, and were also elevated during the i.v. insulin infusion.

Blockade of α_1- or α_2-adrenoceptors in the PVN per se by administration of prazosin or yohimbine had no effect on any of the measured blood parameters compared to vehicle administration. However, subsequent i.v. insulin infusion decreased blood glucose levels to a greater degree. Final glucose levels were 2.5 ± 0.2 mM after α_1-blockade ($p=0.03$ vs vehicle treatment) and 2.7 ± 0.2 mM after α_2-blockade ($p=0.16$ vs vehicle treatment). Glucagon responses were similar to the control group, although the final glucagon level after α_1-blockade was slightly higher (162 ± 8 vs 140 ± 6 pg/ml; $p=0.04$). The adrenaline response was delayed and impaired by α_1- or α_2-blockade, with adrenaline concentrations in both groups only becoming significantly elevated over baseline at t=90 minutes (in the control group, adrenaline levels were significantly elevated already at t=60) and reaching lower final levels (controls: 1598 ± 611 pg/ml; after α_1- or α_2-blockade: 785 ± 223 and 860 ± 287 pg/ml). Noradrenaline also showed a delayed and reduced response, although statistical significance was only obtained between control rats and α_1-blockade ($p<0.05$ at t=60 and t=90), and not between controls and α_2-blockade. Corticosterone levels in plasma increased during the antagonist infusions into the PVN, similarly to the control group. During the insulin infusion corticosterone levels remained elevated also, though at higher concentrations after α-blockade than after control treatment (final levels: control 23 ± 3 μg/dl, α_1-blockade 32 ± 2 μg/dl ($p=0.03$ vs control), α_2-blockade 31 ± 2 μg/dl ($p=0.07$ vs control)).

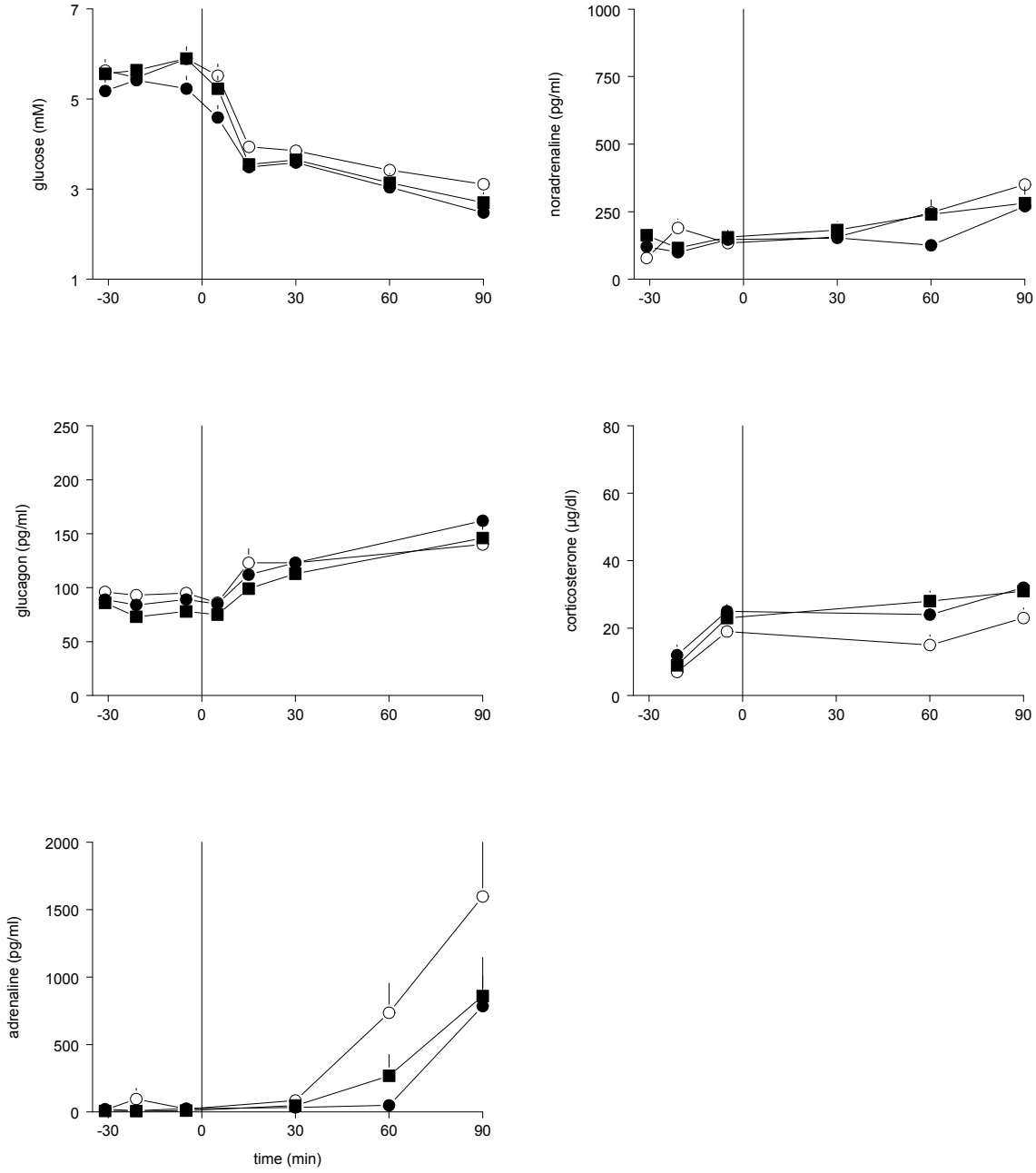

Figure 2. Levels of blood glucose and counterregulatory hormones during hypoglycemia with or without blockade of hypothalamic noradrenergic neurotransmission. From t=-20 to t=-12, the rats received an infusion into the PVN with vehicle (○), the α_1-antagonist prazosin (●), or the α_2-antagonist yohimbine (■). From t=0 to t=90 all rats were infused intravenously with 120 mU.kg^{-1}.min^{-1} insulin.

Half an hour after the end of the insulin infusion, i.e. at t=120 minutes, food was returned to the animals. An hour later the consumed amount of food was recorded, with the results depicted in *Figure 3*. Animals subjected to hypoglycemia without α-adrenergic blockade in the PVN consumed 4.0 ± 0.7 grams of food. Animals pretreated with the α_1-antagonist consumed a greater amount of food after hypoglycemia (5.9 ± 0.5 grams; p=0.051

vs vehicle), while rats pretreated with the α_2-antagonist consumed a slightly smaller amount of food than controls (3.4 ± 0.8 grams; p=0.58 vs vehicle, p=0.01 vs α_1-antagonist).

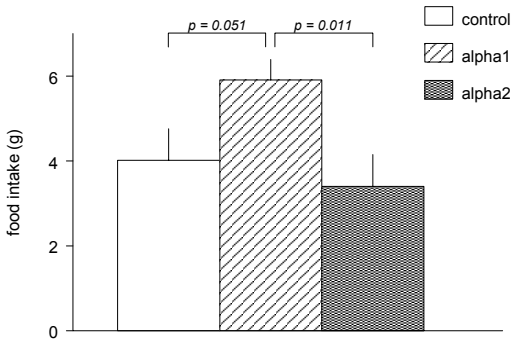

Figure 2. Food intake after hypoglycemia with or without blockade of hypothalamic noradrenergic neurotransmission. Rats were injected in the PVN with vehicle, the α_1-antagonist prazosin, or the α_2-antagonist yohimbine, and then subjected for 90 minutes to insulin-induced hypoglycemia. Food was returned at t=120 minutes and food intake was measured after 1 hour.

Discussion

The current study was undertaken to determine whether α-adrenergic neurotransmission in the PVN is involved in the counterregulatory responses to insulin-induced hypoglycemia. The data revealed that blockade of α-adrenoceptors indeed reduces the sympathoadrenal counterregulatory responses to hypoglycemia, leading to a further reduction in blood glucose levels which is then counteracted by an increase in other counterregulatory responses such as corticosterone, and in individual cases glucagon and food intake.

Intravenous infusion of insulin induced hypoglycemia, which was counteracted by an increase in plasma glucagon and in a later stage by an increase in adrenaline levels. Plasma noradrenaline and corticosterone were also increased but to a lesser extent. The results from the control experiment (where vehicle was infused into the PVN) were similar to our findings in a previous study, in which the same amount of insulin was given to rats without brain cannulas (Chapter 2). Furthermore, when food was returned after the end of the insulin infusion, the animals consumed about four grams of food.

Administration of selective α-adrenoceptor antagonists into the PVN did not by itself affect blood components, except an increase in corticosterone identical to that seen after PVN infusion of vehicle. However, during hypoglycemia clear differences could be observed between treatments. Blood glucose levels decreased more when an α_1- or α_2-adrenoceptor antagonist had been applied into the PVN. Despite that, the responses in adrenaline and noradrenaline were inhibited, with the strongest inhibition seen after blockade of α_1-adrenoceptors – suggesting a predominant role for this noradrenergic receptor subtype in the sympathoadrenal hormonal responses to changes in glucose homeostasis.

In contrast, the glucagon responses were not reduced after PVN α-adrenergic antagonism. Instead, they were identical to or larger than the glucagon response in the control rats. Corticosterone responses were also larger than in controls – assumedly reflecting a compensation for the insufficient sympathoadrenal responses and the further decreased blood glucose levels. These data are very much in line with literature data where

total inactivation of the PVN (by lidocain) did not affect glucagon and corticosterone responses during hypoglycemia, while it reduced the adrenaline and noradrenaline responses (7).

From the abovementioned data, it could be concluded that the activation of the glucagon and corticosterone responses during hypoglycemia is either not regulated by neurotransmission in the PVN, or is also mediated by other mechanisms besides this PVN pathway. Indeed, for glucagon it has been shown that during hypoglycemia, glucagon secretion from the pancreatic alpha-cells is activated via multiple and redundant mechanisms (9, 27). The same might apply for the corticosterone response.

In the animals treated with the α_1-antagonist, food intake after hypoglycemia was higher than in the vehicle treated group. This increased response may be considered a compensatory response for the further reduced blood glucose levels after α_1-blockade in the PVN. Animals treated with the α_2-adrenoceptor antagonist failed to compensate with an increased food intake response, despite lower glucose levels. This suggests that the food intake response to hypoglycemia is mediated by α_2- rather than α_1-adrenoceptors in the PVN, in line with other studies observing that α_2- but not α_1-adrenoceptors are agonistically involved in the regulation of food intake (5, 8, 12).

Taken together, the data reveal that noradrenergic transmission in the PVN plays a role in the counterregulatory responses to insulin-induced hypoglycemia. This is in agreement with the previous chapter and other studies showing activation of noradrenergic projections to the hypothalamus during glucoprivation (1, 19). This noradrenergic neurotransmission seems to be especially important for the sympathoadrenal counterregulatory responses, as seen from the delayed and reduced plasma adrenaline and noradrenaline responses when this neurotransmission is antagonized – while neither the glucagon response nor the corticosterone response were attenuated. Both subtypes of noradrenaline receptors, α_1 and α_2, are involved in these sympathoadrenal responses, although the fact that only α_2-antagonism prevented a compensatory increase in food intake might imply that the two receptor subtypes have different roles in the counterregulation, with α_1-adrenoceptors perhaps being more involved in regulation of the hormonal responses, while α_2-adrenoceptors seem to mediate the behavioral food intake response.

In conclusion, our data confirm the findings in Chapter 4 that the noradrenergic pathways projecting to the PVN play an important role in the counterregulatory responses to insulin-induced hypoglycemia in rats.

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Section III – Recurrent hypoglycemia

The most exciting phrase to hear in science, the one that heralds new discoveries, is not "Eureka!" but "That's funny..."
- Isaac Asimov